Cell Injury, Repair, Aging and Apoptosis

Nitric Oxide Is an Important Mediator of Renal Tubular Epithelial Cell Death *in Vitro* and in Murine Experimental Hydronephrosis

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Macrophages play a pivotal role in tissue injury and fibrosis during renal inflammation. Although macrophages may induce apoptosis of renal tubular epithelial cells, the mechanisms involved are unclear. We used a microscopically quantifiable co-culture assay to dissect the cytotoxic interaction between murine bone marrow-derived macrophages and Madin-Darby canine kidney cells and primary murine renal tubular epithelial cells. The induction of tubular cell apoptosis by cytokine-activated macrophages was reduced by inhibitors of nitric oxide synthase whereas tubular cell proliferation was unaffected. Furthermore, cytokine-activated macrophages derived from mice targeted for the deletion of inducible nitric oxide synthase were noncytotoxic. We then examined the role of nitric oxide *in vivo* by inhibiting inducible nitric oxide synthase in the model of murine experimental hydronephrosis. L-N^6-(1-iminoethyl)-lysine was administered in the drinking water between days 5 and 7 after ureteric obstruction. Macrophage infiltration was comparable between groups, but treatment significantly inhibited tubular cell apoptosis at day 7. Tubular cell proliferation was unaffected. Inducible nitric oxide synthase blockade also reduced interstitial cell apoptosis and increased collagen III deposition. These data indicate that nitric oxide is a key mediator of macrophage-directed tubular cell apoptosis *in vitro* and *in vivo* and also modulates tubulointerstitial fibrosis. *(Am J Pathol 2006, 169:388–399; DOI: 10.2353/ajpath.2006.050964)*

Macrophages are remarkably versatile cells that play a major role in many key biological processes including host defense, wound healing, development, tissue remodeling, acute inflammation, and the clearance of apoptotic cells.[1–5] Macrophages may induce apoptosis of host cells and this is beneficial in the context of developmental sculpting of tissues[4,6,7] but is usually deleterious during renal inflammation.[8–10] Recent work indicates that macrophages may induce tubular epithelial cell apoptosis in both immunological and nonimmunological tubulointerstitial inflammation.[11–14] Tesch and colleagues[11] induced nephrotoxic glomerulonephritis in mice targeted for the deletion of monocyte chemoattractant protein-1 (MCP-1). The resultant diminished tubulointerstitial macrophage infiltrate was associated with a reduction in tubular epithelial cell apoptosis. This finding was reinforced by our recent work that used conditional macrophage ablation in progressive nephrotoxic glomerulonephritis because macrophage ablation significantly reduced the level of tubular epithelial cell apoptosis.[14] The role of macrophages in the nonimmunological model of experimental hydronephrosis induced by unilateral ureteric obstruction has also been studied.[12,13] Lenda and colleagues[12] obstructed the kidneys of colony-stimulating-factor-1 (CSF-1)-deficient mice and demonstrated a reduced interstitial macrophage infiltrate associated with a reduced level of tubular epithelial cell death. Lange-Sperandio and colleagues[13] obstructed the kidneys of triple E-, P-, and L-selectin knockout mice or wild-type control mice within the first 48 hours after birth. Triple selectin knockout mice exhibited diminished tubulointerstitial macrophage infiltration and reduced levels of tubular epithelial cell death compared to control mice.

Although tubular cell apoptosis is invariably present during renal injury, excessive levels of tubular cell death are highly undesirable and result in renal tubular atrophy, hypocellular scarring and eventual organ failure.[15] Indeed, the tubulointerstitium of the kidney plays an important role in all renal diseases irrespective of the nature of the original injury[16] because there is a striking correlation between the severity of the tubulointerstitial changes in human biopsies and the subsequent development and progression of chronic renal failure to end-stage renal failure requiring dialysis.[17]

Currently, despite the documented correlation between the severity of macrophage infiltration and the level of tubular epithelial cell apoptosis,[12–14] there is scant data regarding the mechanisms involved in macrophage-mediated tubular cell apoptosis. Inflammatory macrophages produce myriad proapoptotic mediators that may kill neighboring cells, including nitric oxide (NO), tumor necrosis factor-α (TNF-α), as well as Fas ligand (FasL).[18–20] Previous *in vitro* studies addressing this issue used both macrophage and murine tubular cell lines[21] with cytokine-activated J774 macrophages inducing apoptosis of murine PKSV-PR proximal tubular cells. This study and previous work by Tesch and colleagues,[11] however, did not determine the nature of the macrophage death effector although no role for TNF-α, FasL, or transforming growth factor-β was demonstrable.[21]

In this study we have used a well-established microscopically quantifiable co-culture assay[18] and the model of experimental hydronephrosis[12,13,22] to examine the cytotoxic mechanism underlying macrophage-mediated tubular epithelial cell death *in vitro* and *in vivo*. We demonstrate an important role for macrophage-derived NO in the induction of tubular cell apoptosis *in vitro* and during tubulointerstitial inflammation *in vivo*. In addition, our data reinforces a role for NO in modulating tubulointerstitial fibrosis and scarring.

Materials and Methods

Materials

Tissue culture reagents were purchased from Life Technologies (Paisley, UK). Tissue culture plastics were obtained from Costar (Loughborough, Leicestershire, UK) and Falcon (Runcorn, Cheshire, UK). Cytokines were purchased from R&D Systems (Abingdon, Oxon, UK) and Peprotech EC Ltd. (London, UK). L-N^6-(1-iminoethyl)-lysine (L-NIL) and the control inactive isomer D-N^6-(1-iminoethyl)-lysine (D-NIL) were purchased from Fluorochem Ltd. (Old Glossop, Derbyshire, UK). All other reagents were from Sigma-Aldrich Co. Ltd. (Poole, UK) unless otherwise stated.

Experimental Animals

Inducible nitric oxide synthase (iNOS) knockout[23] and wild-type control mice were obtained from B and K Universal (Hull, UK). iNOS knockout and wild-type mice were

on the 129/sv background. CB7BL/6 and FVB/N mice were bred at the University of Edinburgh.

Preparation of Bone Marrow-Derived Macrophages

Bone marrow-derived macrophages were used in these studies and were prepared from FVB/N mice, C57BL/6, or iNOS knockout and wild-type mice as described previously.[18] Briefly, bone marrow was isolated from femurs by standard sterile techniques and matured for 7 days in sterile Teflon wells in Dulbecco's modified Eagle's medium (DMEM)/F12 medium with 10% heat inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% L929 cell-conditioned medium as a source of M-CSF. Macrophages were greater than 98% positive for the macrophage marker F4/80 by flow cytometry.

Renal Tubular Epithelial Cell Culture

Madin-Darby canine kidney (MDCK) cells (a gift from Dr. J. Davie, University of Edinburgh) were cultured as described previously.[24] Briefly, cells were grown as a monolayer culture in 75-cm^2 culture flasks and maintained with Eagle's minimum essential medium containing 1% nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS in a humidified atmosphere of 5% CO_2 at 37°C. Murine primary tubular epithelial (PTE) cells were derived from the kidneys of C57BL/6 mice after microdissection and brief collagenase digestion.[25] PTE cells were grown as a monolayer culture in 25-cm^2 culture flasks and maintained with DMEM/F12 medium containing insulin (10 μg/ml), transferrin (5.5 μg/ml), selenium (5 ng/ml), epidermal growth factor (25 ng/ml), and dexamethasone (36 ng/ml). PTE cells were cytokeratin-positive and vimentin-negative by immunochemistry.

Co-Culture Studies

MDCK or PTE cells were prelabeled with fluorescent CellTracker Green whereas in some experiments mature macrophages (7 to 10 days) were prelabeled with fluorescent CellTracker Orange (both CellTracker dyes obtained from Molecular Probes, Eugene, OR). Cells were washed with serum-free medium and incubated for 30 minutes (macrophage) or 1 hour (epithelial cells) in serum-free medium containing the respective CellTracker dye at a concentration of 5 ng/ml. Cells were washed in medium containing 10% FCS to remove unbound CellTracker dye. Epithelial cells were then trypsinized and added to 48-well plates at a density to cover 60 to 70% of the well surface: 1×10^4 MDCK cells/well and 1.5×10^4 PTE cells/well. Wells were washed after 2 to 4 hours to remove nonadherent cells. Macrophages were added to epithelial cells at a ratio of two macrophages to one epithelial cell. MDCK experiments were conducted in DMEM/F12 medium containing 10% FCS, and PTE cell

experiments were conducted in PTE cell medium containing 0.1% FCS.

Selected co-cultures were activated with lipopolysaccharide (LPS) (1 μg/ml) and murine interferon-γ (IFN-γ) (100 U/ml), with nonactivated co-cultures being exposed to medium alone. After 24 hours of incubation, the undisturbed co-cultures underwent *in situ* fixation with formaldehyde (4% final concentration) to ensure retention of apoptotic cells.[18] Fixed co-cultures were then stained with Hoechst 33342 at 1 μg/ml in phosphate-buffered saline (PBS) for 15 minutes. Using inverted fluorescent microscopy, nonoverlapping fields from each well were randomly and blindly chosen so that at least 200 epithelial cells were counted per well. Apoptotic epithelial cells were identified by their green condensed cytoplasm and pyknotic nuclei. Mitotic epithelial cells were discernible by their characteristic chromatin pattern. The number of apoptotic or mitotic epithelial cells was counted and expressed as apoptotic or mitotic cells per high-power field or as a percentage of the total number of epithelial cells (percent apoptosis). All experimental conditions were performed in triplicate and experiments performed on at least three separate occasions. Macrophage and primary PTE cell cultures were derived from at least three different animals.

The Model of Experimental Hydronephrosis

Experimental hydronephrosis was induced by performing unilateral ureteric obstruction.[26] The left ureter of age-matched male FVB/N mice was ligated under inhalational anesthesia. Mice were administered either L-NIL or the control inactive isomer D-NIL in the drinking water (1 mg/ml) for 48 hours before sacrifice at day 7 (n = seven to eight per group).[27,28] The removed kidneys were cut longitudinally and fixed in either 10% buffered formalin or methyl Carnoy's solution (60% methanol, 30% chloroform, and 10% acetic acid) and embedded in paraffin. All experiments were performed in accordance with the UK Government Home Office regulations.

Renal Morphology and Immunohistochemistry

To examine renal histology, 4-μm sections were stained with periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. Tubulointerstitial macrophage infiltration was quantified after immunostaining for the murine macrophage marker F4/80. Briefly, methyl Carnoy's fixed tissue sections were deparaffinized, rehydrated in ethanol, and incubated in 3% H_2O_2 in methanol to block endogenous peroxidase activity. Tissue sections were then incubated with rat monoclonal antibody (IgG2b) directed against mouse F4/80 (1/1000 dilution; Caltag Laboratories, Northampton, UK) at 4°C overnight, followed by a mouse-adsorbed biotinylated rabbit anti-rat IgG (1/1000 dilution; Vector Laboratories, Peterborough, UK) at room temperature for 30 minutes. For iNOS immunostaining, tissue sections were incubated with a polyclonal rabbit antibody (1/50 dilution; Abcam Laboratories, Cambridge, UK) at 4°C overnight, followed by a

biotinylated goat anti-rabbit IgG (1/300 dilution; DakoCytomation, Glostrup, Denmark) at room temperature for 30 minutes. The tubulointerstitial myofibroblast population was quantified after immunostaining for the myofibroblast marker α-smooth muscle actin. Tissue sections were incubated with monoclonal mouse anti-human α-smooth muscle actin that cross-reacts with murine α-smooth muscle actin [clone 1A4 (IgG2a), 1/1000 dilution; Sigma-Aldrich Co. Ltd., Poole, UK] at 4°C overnight, followed by a biotinylated rat-anti-mouse IgG2a (1/100 dilution; Zymed Laboratories, San Francisco, CA) at room temperature for 30 minutes. Tubulointerstitial fibrosis was quantified after immunostaining for collagen III. Tissue sections were incubated with goat-anti-human type III collagen antibody, which also detects murine type III collagen (1/50 dilution; Cambridge BioScience Ltd., Cambridge, UK) at 4°C overnight, followed by a biotinylated rabbit-anti-goat IgG (1/100 dilution; Vector Laboratories) at room temperature for 30 minutes.

After washing in PBS, sections were incubated in horseradish peroxidase-conjugated avidin D (1/2000 dilution; Vector Laboratories) at room temperature for 20 minutes. Color was developed using diaminobenzidine as the chromogen and counterstained with methyl green or hematoxylin. An irrelevant isotype control primary antibody served as negative control. Positive control tissue included sections from diseased mice that were known to express F4/80-positive macrophages or exhibit significant renal scarring. Interstitial macrophage infiltration was quantified in a blinded manner by analyzing 10 sequentially selected nonoverlapping fields of renal cortex of F4/80-stained sections at ×100 magnification using computer-assisted image analysis (ImageJ 1.30h; National Institutes of Health, Bethesda, MD; *http://rsb.info. nih.gov/ij/Java1.3.1_03*).[14,29,30] Macrophage infiltration was expressed as the percentage of tissue surface area positive for F4/80 staining.[14,31] Tubulointerstitial collagen III deposition was quantified in a similar manner while myofibroblast accumulation was expressed as the percentage of tissue surface area positive for α-smooth muscle actin staining excluding blood vessels.

Double-Immunofluorescence Staining for iNOS and F4/80

Methyl Carnoy's fixed tissue sections were deparaffinized, rehydrated, and incubated in 3% H_2O_2 in methanol to block endogenous peroxidase activity. Nonspecific binding was blocked by incubation in 2% goat serum, 1% bovine serum albumin, 0.1% Triton X-100, and 0.05% Tween 20 for 30 minutes at room temperature. Tissue sections were then sequentially incubated in the following antibodies: 1) polyclonal rabbit anti-iNOS (1/50 dilution; BD Biosciences Pharmingen, Oxford, UK); 2) AlexaFluor-488-conjugated goat anti-rabbit IgG (1/300 dilution; Molecular Probes); 3) rat anti-mouse F4/80 (IgG2b, 1/1000 dilution; Caltag Laboratories, Northampton, UK); 4) mouse-adsorbed biotinylated rabbit anti-rat IgG (1/1000 dilution, Vector Laboratories); and 5) Alexa Fluor-568-conjugated streptavidin (1/300 dilution, Molec-

ular Probes). Primary antibodies were incubated overnight at 4°C, with remaining incubations being performed at room temperature for 30 minutes. After each incubation step tissue sections were washed three times in Tris-buffered saline (pH 7.6) for 5 minutes. To assess the specificity of the immunostaining, tissue sections were incubated with nonimmune rabbit or rat IgG in place of the primary anti-iNOS or anti-F4/80 antibodies and then processed under identical conditions. Tissue sections were mounted with anti-fade mounting medium (Vector Laboratories), and double-immunofluorescent staining was analyzed by inverted fluorescent microscopy.

Detection of Apoptosis and Proliferation

Apoptotic cells were detected by the terminal dUTP nick-end labeling (TUNEL) assay as previously described.[32] Briefly, 4-μm formalin-fixed tissue sections were deparaffinized and rehydrated in ethanol followed by an antigen retrieval step comprising of boiling in 0.01 mol/L sodium citrate buffer for 2 minutes. Sections were then incubated with proteinase K (6.2 μg/ml), followed by TdT (300 enzyme U/ml; Amersham Pharmacia Biotech) and Bio-14-dATP (0.94 nmol/L; Gibco BRL, Life Technologies, Paisley, Scotland). Biotinylated ATP was detected using the RTU Vectastain Elite ABC Reagent (Vector Laboratories) and slides were counterstained with methyl green and eosin. As a positive control, slides were pretreated with DNase I (20 Kunitz U/ml; Roche Molecular Biochemicals, Lewes, UK). Cells were regarded as TUNEL-positive if they exhibited stained nuclei with an apoptotic morphology. Tubular and interstitial cell apoptosis was quantified in a blinded manner by counting the number of TUNEL-positive tubular and interstitial cells in 20 to 25 sequentially selected nonoverlapping fields of renal cortex at ×400 magnification. Data were expressed as the mean number ± SEM per high-power field.

PAS-stained tissue sections were used to quantify proximal and distal tubular cell proliferation because proximal tubular cells exhibit a characteristic PAS-positive luminal brush border.[33] Mitotic cells were readily identifiable, and proximal and distal cell proliferation were calculated in a blinded manner by counting the number of mitotic proximal and distal tubular epithelial cells in 20 to 25 sequentially selected nonoverlapping fields of renal cortex at ×400 magnification and expressed as the mean number ± SEM per high-power field.

Statistical Analysis

All results are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 3.02/Instat 1.1 (GraphPad Software, San Diego, CA). The Student's *t*-test was used for comparisons involving two groups, and statistical differences among multiple groups of data were assessed by one-way analysis of variance followed by a Newman-Keuls post hoc test. Results are considered significant at $P < 0.05$.
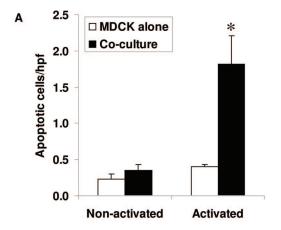
Results

Cytokine-Activated Macrophages Induce MDCK Cell Apoptosis

Nonactivated macrophages did not induce significant MDCK cell apoptosis in co-culture studies, thereby indicating that macrophages are not inherently cytotoxic (Figure 1A; 1.49 ± 0.3% versus 0.96 ± 0.3% apoptosis, nonactivated co-cultures versus control MDCK cells; $P > 0.05$). In addition, treatment of MDCK cells with LPS and IFN-γ had no effect on the level of MDCK cell apoptosis (1.42 ± 0.3% versus 0.96 ± 0.3% apoptosis, cytokine-activated MDCK cells versus nonactivated MDCK cells; $P > 0.05$). However, MDCK cells in co-cultures activated with LPS and IFN-γ exhibited significantly increased levels of apoptosis after 24 hours (Figure 1A; 12.3 ± 4.1% versus 1.49 ± 0.3% apoptosis; cytokine-activated co-cultures versus nonactivated co-cultures; $P < 0.01$). Apoptotic MDCK cells exhibited characteristic cytoplasmic condensation (Figure 1B) and nuclear pyknosis (Figure 1C).

Macrophage-Derived NO Is an Important Mediator of MDCK Cell Apoptosis but Is Not Implicated in the Inhibition of MDCK Cell Proliferation

Previous data indicate that macrophage-derived NO is involved in the cytotoxicity of cytokine-activated macrophages toward tumor cells[34] and glomerular mesangial cells.[18] We therefore performed co-cultures with macrophages derived from either iNOS wild-type or iNOS knockout mice. Co-cultures of activated iNOS wild-type macrophages and MDCK cells exhibited a 3.5-fold higher level of MDCK cell apoptosis compared to co-cultures of MDCK cells with activated iNOS knockout macrophages (Figure 2A; 4.6 ± 0.9% versus 1.14 ± 0.4% apoptosis; activated co-cultures with iNOS wild-type macrophages versus activated co-cultures with iNOS knockout macrophages; $P < 0.001$). In addition, cytokine-activated iNOS knockout macrophages did not induce apoptosis above the background level evident in cytokine-treated target cells alone, thereby suggesting a key role for macrophage-derived NO in the induction of MDCK death (1.14 ± 0.4% versus 0.69 ± 0.04% apoptosis; activated co-cultures with iNOS knockout macrophages versus nonactivated co-cultures with iNOS knockout macrophages; $P > 0.05$). Furthermore, the cytotoxic effect of activated iNOS wild-type macrophages was completely abrogated by the inclusion of the noncompetitive NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME, 200 μmol/L) thereby reinforcing the importance of NO as a death effector in cytokine-activated co-cultures (Figure 2A; 0.92 ± 0.1% versus 4.6 ± 0.9% apoptosis; activated co-culture with L-NAME versus activated co-culture; $P < 0.001$). Importantly, inclusion of the control isomer *N*-nitro-D-arginine methyl ester (D-NAME, 200 μmol/L) did not prevent the cytotoxic action of cytokine-activated iNOS wild-type macrophages (Fig-
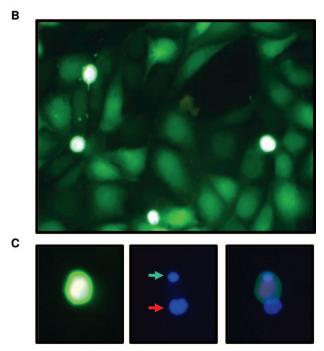
Figure 1. Cytokine-activated macrophages induce MDCK cell apoptosis. **A:** MDCK cells were cultured alone or in the presence of mature murine bone marrow-derived macrophages in the presence or absence of LPS and IFN-γ. After a 24-hour incubation the level of MDCK cell apoptosis is determined by *in situ* fixation of cultures with formaldehyde and fluorescence microscopy after staining with Hoechst. Cytokine-activated macrophages induce significant MDCK cell death. *$P < 0.001$ versus nonactivated co-culture (data from experiments with macrophages from four different mice). **B:** Low-power view of cytokine-activated co-culture of macrophages (unlabeled) and Cell-Tracker green-labeled MDCK cells after 24 hours. Apoptotic MDCK cells are visible as bright cells exhibiting cytoplasmic condensation. **C:** The bright rounded apoptotic MDCK cell exhibits nuclear pyknosis (**green arrow**); a classical feature of apoptosis. The nucleus of an adjacent unlabeled macrophage is also evident (**red arrow**). The merged image demonstrates the proximity of the macrophage to the MDCK cell.
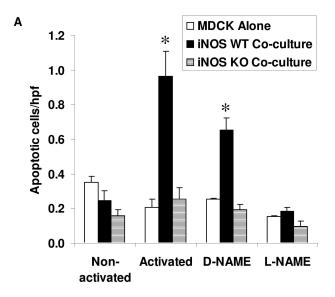

Figure 2. Macrophage-derived NO is an important mediator of MDCK cell apoptosis but is not involved in the inhibition of MDCK cell proliferation. **A:** MDCK cells were cultured alone or in the presence of bone marrow-derived macrophages derived from either iNOS wild-type (WT) or knockout (KO) mice. Cultures were activated with LPS and IFN-γ in the presence or absence of the NOS inhibitor L-NAME (200 μmol/L) or control D-NAME (200 μmol/L). iNOS knockout macrophages are not cytotoxic under any conditions whereas the cytotoxicity of iNOS wild-type macrophages is completely abrogated by pharmacological inhibition of NO production. *$P < 0.05$ versus nonactivated iNOS wild-type macrophage co-cultures (data from experiments with macrophages from three different mice). **B:** MDCK cells were cultured alone or in the presence of mature bone marrow-derived macrophages derived from either iNOS wild-type or knockout mice. Cultures were activated with LPS and IFN-γ in the presence or absence of the NOS inhibitor L-NAME. Inhibition of MDCK cell proliferation by macrophages is independent of cytokine activation, macrophage genotype, or pharmacological inhibition of NO production. *$P < 0.05$ versus MDCK cells alone (data from experiments with macrophages from three different mice).

ure 2A; 4.4 ± 0.3% versus 4.6 ± 0.9% apoptosis; activated co-cultures with D-NAME versus activated co-cultures; $P > 0.05$).

MDCK cell proliferation was significantly inhibited by co-culture with macrophages, with the anti-proliferative effect being independent of macrophage activation by LPS and IFN-γ (Figure 2B). Furthermore, this anti-prolif-
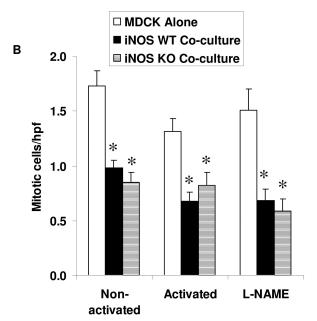
erative effect was unaffected by the inclusion of the NOS inhibitor L-NAME in the co-culture or the use of iNOS wild-type and knockout macrophages (Figure 2B). This indicates, in contrast to previous work using mesangial

cells,[18] that NO was not involved in the modulation of MDCK cell proliferation. No difference was evident in total cell number in these experiments (data not shown).

Macrophage-Derived NO Induces Apoptosis in Murine PTE Cells

Although MDCK cells are a well-established tubular cell line, they are of canine origin and may therefore not be susceptible to other putative murine macrophage-derived death effectors such as FasL and TNF-α. We therefore determined whether cytokine-activated macrophages exerted similar effects on murine PTE cells. Treatment of PTE cells with LPS and IFN-γ did not induce significant apoptosis (Figure 3A). Cytokine activation of co-cultures induced a significant threefold increase in PTE cell apoptosis compared to nonactivated co-cultures (Figure 3A; $5.28 \pm 0.7\%$ versus $1.08 \pm 0.3\%$ apoptosis; activated co-cultures versus nonactivated co-cultures; $P < 0.001$). In addition, inclusion of the NOS inhibitor L-NAME reduced tubular cell death to baseline levels (Figure 3A; $5.28 \pm 0.7\%$ versus $1.47 \pm 0.3\%$ apoptosis; activated co-cultures versus activated co-cultures treated with L-NAME; $P < 0.001$). Furthermore, although iNOS wild-type macrophages were significantly cytotoxic (Figure 3B; $3.26 \pm 0.4\%$ versus $0.72 \pm 0.3\%$ apoptosis; activated co-cultures with iNOS wild-type macrophages versus nonactivated co-cultures with iNOS wild-type macrophages; $P < 0.001$), cytokine-activated iNOS knockout macrophages did not induce significant apoptosis of PTE cells (Figure 3B; $0.57 \pm 0.4\%$ versus $0.33 \pm 0.1\%$ apoptosis; activated co-cultures with iNOS knockout macrophages versus nonactivated co-cultures with iNOS knockout macrophages; $P > 0.05$).

In contrast to MDCK cells, incubation of PTE cells with macrophages in the presence or absence of LPS and IFN-γ had no effect on the level of PTE cell proliferation (data not shown). It should, however, be noted that PTE cells exhibit a low level of proliferation compared to MDCK cells. Although there was a trend to a reduced total cell number in activated co-cultures, this did not reach statistical significance (data not shown). We also noted evidence of phagocytosis of apoptotic PTE cells by macrophages in this primary cell co-culture system (Figure 3C) unlike co-cultures of macrophages with MDCK cells. The effect of phagocytosis would be predicted to reduce the levels of free apoptotic cells evident, and this may explain why a lower level of apoptotic PTE cells was detected compared to MDCK cells. Interestingly, in accordance with the increased level of PTE cell apoptosis in activated co-cultures of iNOS wild-type macrophages compared to iNOS knockout macrophages, we noted an increased percentage of macrophages containing phagocytosed apoptotic PTE cells (1.2 ± 0.79 macrophages/high-power field versus 0.3 ± 0.25 macrophages/high-power field: iNOS wild-type macrophages versus iNOS knockout macrophages). In these studies we did not find any significant difference between experiments involving the co-culture of C57BL/6 PTE cells with either allogeneic FVB/N macrophages or syngeneic
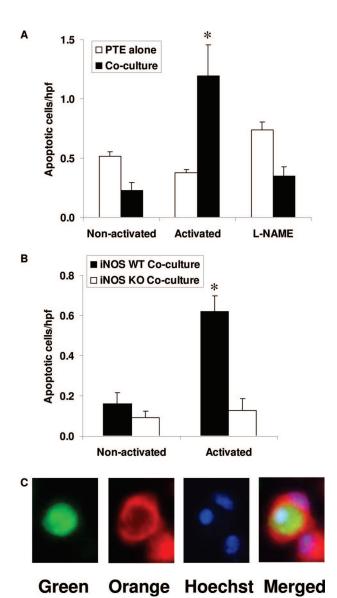

Green   Orange   Hoechst   Merged

Figure 3. Cytokine-activated macrophages induce apoptosis in primary murine tubular epithelial cells (PTEs). **A:** PTE cells were cultured in the presence of mature murine bone marrow-derived macrophages. Selected cell cultures were activated with LPS and IFN-γ in the presence or absence of the NOS inhibitor L-NAME (200 μmol/L). *$P < 0.05$ versus activated co-cultures in the presence or absence of L-NAME (data from experiments with cells from three different mice). **B:** PTE cells were cultured in the presence of mature bone marrow-derived macrophages derived from either iNOS wild-type or knockout mice. Selected cell cultures were activated with LPS and IFN-γ. Cytokine activation of iNOS wild-type macrophages induced PTE cell apoptosis whereas activated iNOS knockout macrophages were noncytotoxic. *$P < 0.05$ versus nonactivated co-culture (data from experiments with cells from five different mice). **C:** Photomicrographs from a co-culture of fluorescently labeled macrophages (CellTracker orange) and PTE cells (CellTracker green) demonstrating evidence of macrophage recognition and phagocytosis of apoptotic tubular cells. The merged image demonstrates a condensed green apoptotic tubular cell with a pyknotic nucleus that has been ingested by a CellTracker orange-labeled macrophage.

C57BL/6 macrophages (0.58 ± 0.1 apoptotic cells per high-power field versus 0.42 ± 0.06 apoptotic cells per high-power field; activated co-cultures with C57BL/6 PTE cells and FVB/N macrophages versus activated co-cultures with C57BL/6 PTE cells and C57BL/6 macrophages; $P > 0.05$).
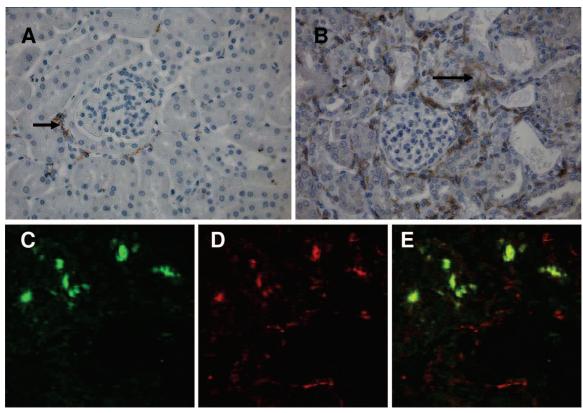
Figure 4. Obstructed kidneys exhibit tubulointerstitial infiltration with macrophages and iNOS-positive cells. Tissue sections were immunostained for the macrophage marker F4/80 and/or iNOS. **A:** Normal nonmanipulated kidneys exhibit occasional F4/80-positive resident macrophages (**arrow**). **B:** In contrast, obstructed kidneys exhibit a prominent interstitial population of infiltrating F4/80-positive macrophages (**arrow**). **C–E:** Fluorescent photomicrographs showing double-immunofluorescent staining of iNOS and F4/80 in day 7 obstructed kidney: iNOS-positive cells stained green (**C**), F4/80-positive interstitial macrophages stained red (**D**), and merged image demonstrating co-localization (yellow) of iNOS and F4/80 indicating the presence of infiltrating macrophages expressing iNOS (**E**).

Pharmacological Blockade of iNOS by L-NIL Reduces Tubular Epithelial Cell Apoptosis in Experimental Hydronephrosis

We then sought a role for iNOS-derived NO in the tubular cell apoptosis evident during tubulointerstitial inflammation *in vivo*. We used L-NIL, a specific irreversible iNOS inhibitor, to pharmacologically inhibit the enzyme iNOS in the model of experimental hydronephrosis, a model characterized by marked tubulointerstitial macrophage infiltration and tubular epithelial cell death.[22,35] We induced experimental hydronephrosis in FVB/N mice and administered L-NIL in the drinking water[27,28] between day 5 and day 7, at which time the obstructed kidneys were removed for histological analysis. Administration of the inactive isomer D-NIL served as control. Normal kidneys exhibited occasional scattered F4/80-positive resident macrophages whereas obstructed kidneys developed a prominent macrophage infiltrate in the tubulointerstitium (Figure 4, A and B; $0.32 \pm 0.04\%$ versus $3.9 \pm 0.9\%$ F4/80-positive surface area; normal kidney versus day 7 obstructed kidney; $P < 0.001$). In addition, iNOS-positive macrophages were evident within the tubulointerstitium (Figure 4, C–E). Pharmacological inhibition of iNOS did not have any significant effect on the level of macrophage infiltration ($3.2 \pm 0.7\%$ versus $3.9 \pm 0.9\%$ F4/80-positive surface area; L-NIL versus control; $P > 0.05$). Tubular cell

apoptosis and proliferation was determined by TUNEL and PAS staining, respectively (Figure 5). Administration of L-NIL significantly reduced the level of tubular epithelial cell apoptosis compared to controls (Figure 6A). In contrast, the administration of L-NIL did not affect the level of tubular cell proliferation in obstructed kidneys (Figure 6B).

Pharmacological Blockade of iNOS by L-NIL Reduces Interstitial Cell Apoptosis and Increases Tubulointerstitial Fibrosis in Experimental Hydronephrosis

Interstitial cell apoptosis and proliferation were determined by TUNEL and PAS staining, respectively (Figure 5). The administration of L-NIL also resulted in a significant reduction in the level of interstitial cell apoptosis (Figure 7A) with no effect on interstitial cell proliferation being evident (Figure 7B). Tubulointerstitial fibrosis was assessed by immunohistochemical staining for collagen III, which is deposited by fibroblasts and is a representative collagen of the scarred and injured tubulointerstitium.[36] Careful morphometric analysis of collagen III immunostaining indicated that pharmacological blockade of the enzyme iNOS resulted in increased collagen III deposition (Figure 8). We stained sections for α-smooth
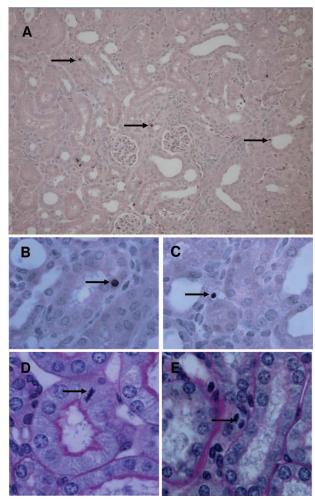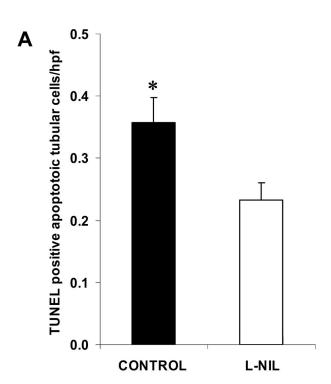
Figure 5. Obstructed kidneys exhibit apoptosis and proliferation of tubular epithelial and interstitial cells. Tissue sections underwent TUNEL staining to detect apoptotic cells whereas cells undergoing mitosis were identified on PAS-stained tissue sections. **A:** Low-power view of obstructed kidneys demonstrating scattered TUNEL-positive apoptotic cells (**arrows**). **B:** High-power view of a TUNEL-positive apoptotic tubular epithelial cell (**arrow**). **C:** High-power view of a TUNEL-positive apoptotic interstitial cell (**arrow**). **D:** High-power view of a tubular epithelial cell undergoing mitosis (**arrow**). **E:** High-power view of an interstitial cell undergoing mitosis (**arrow**). Original magnifications: ×200 (**A**); ×1000 (**B–E**).

muscle actin, which is expressed by tubulointerstitial myofibroblasts.[26] The area of the renal tubulointerstitium occupied by α-smooth muscle actin staining was assessed morphometrically as an indirect measure of the tubulointerstitial myofibroblast population. This analysis indicated no significant difference in α-smooth muscle actin staining after short-term blockade of iNOS (3.1 ± 0.4% versus 2.8 ± 0.6% α-smooth muscle actin staining; L-NIL treatment versus control; $P > 0.05$).

Discussion

The main finding of this study is that, despite the fact that inflammatory macrophages may produce myriad pro-apoptotic mediators,[18–20] NO is the key mediator of macrophage-induced tubular epithelial cell apoptosis *in vitro* and plays a prominent role *in vivo* during tubulointerstitial

Figure 6. Pharmacological blockade of iNOS reduces tubular cell apoptosis but does not affect tubular cell proliferation after ureteric obstruction. Mice were administered L-NIL, an irreversible pharmacological inhibitor of the enzyme iNOS, or the isomeric control D-NIL, and the obstructed kidneys were removed at day 7. **A:** The level of tubular cell apoptosis in obstructed kidneys is significantly reduced after the administration of L-NIL. *$P < 0.05$ versus control (eight mice per group). **B:** The level of tubular cell proliferation in obstructed kidneys is unaffected after the administration of L-NIL (n = seven to eight mice per group).
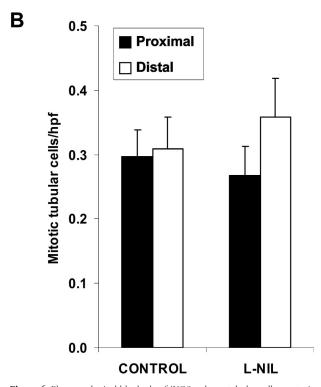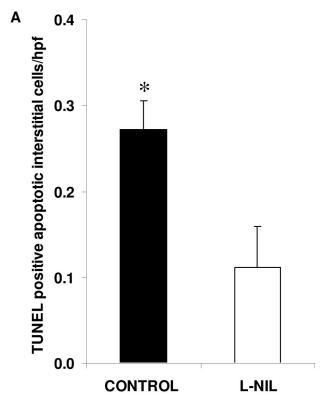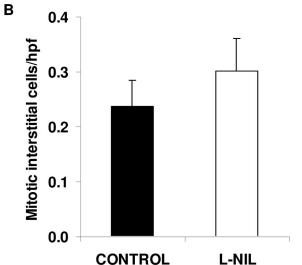
A

B

Figure 7. Pharmacological blockade of iNOS reduces interstitial cell apoptosis but does not affect interstitial cell proliferation after ureteric obstruction. Mice were administered L-NIL, an irreversible pharmacological inhibitor of the enzyme iNOS, or the isomeric control D-NIL, and the obstructed kidneys were removed at day 7. **A:** The level of interstitial cell apoptosis in obstructed kidneys is significantly reduced after the administration of L-NIL. *$P < 0.05$ versus control (eight mice per group). **B:** The level of interstitial cell proliferation in obstructed kidneys is unaffected after the administration of L-NIL (*n* = seven to eight mice per group).
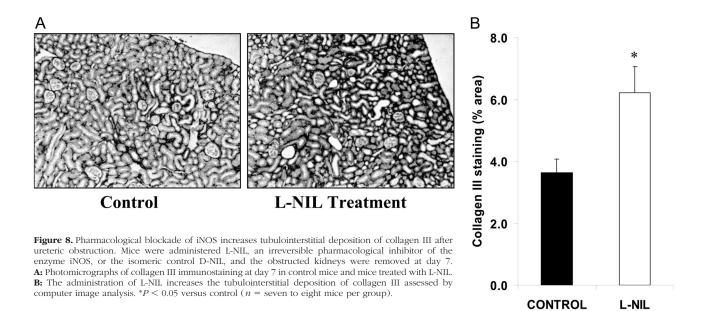
inflammation. Our data indicates that nonactivated macrophages per se are not inherently cytotoxic whereas cytokine-activated macrophages induce significant apoptosis of MDCK cells, a well-characterized renal tubular cell line. Studies using both pharmacological inhibitors of NO production and macrophages derived from either iNOS knockout or wild-type mice indicate that macro-

phage cytotoxicity *in vitro* is markedly dependent on iNOS-derived NO. Importantly, similar results were obtained in an entirely primary cell co-culture system using murine primary renal tubular epithelial cells. These data are in accordance with previous work indicating that NO is an important mediator of macrophage cytotoxicity toward renal mesangial cells and tumor cells.[18,34] Interestingly, we noted that the level of PTE cell apoptosis evident in these studies was less than the level of MDCK cell apoptosis. Although this may reflect a variety of factors including the different species of origin and the proliferation status of the cells, our data suggest that macrophage phagocytosis of apoptotic PTE cells but not MDCK cells may be at least partly responsible because apoptotic cells may be rapidly recognized, ingested, and degraded.[37]

Despite the fact that previous work has indicated the involvement of NO in the macrophage induced apoptosis of mesangial cells[18] and vascular smooth muscle cells,[38] other death effectors such as TNF-α[12,19,39]; and FasL[35,38,40] have also been implicated. In our study, however, pharmacological inhibition of NO production or the use of iNOS-deficient macrophages resulted in a complete abrogation of tubular cell apoptosis compared to control co-cultures, thereby suggesting that NO is the dominant mediator of tubular epithelial cell death induced by cytokine-activated macrophages *in vitro*. This suggests that the presence of significant additional *in vitro* proapoptotic mechanisms is unlikely and that macrophage generation of NO is critically important. Previous *in vitro* studies have suggested macrophage production of a soluble proapoptotic factor capable of inducing tubular cell death,[11,12,21] and this may reflect the use of different cells and assays in these studies.

The effect of macrophages on the level of target cell proliferation varied according to the nature of the target cell. The proliferation of MDCK cells was reduced by incubation with nonactivated or cytokine-activated macrophages. Bone marrow-derived macrophages secrete significant levels of transforming growth factor-β and this cytokine has been documented to inhibit MDCK cell proliferation.[24,41] In contrast, bone marrow-derived macrophages exerted no significant effects on PTE cell proliferation although this may reflect the lower level of proliferation of these primary cells.

Because *in vitro* studies suggested a key role for macrophage-derived NO, we examined the effects of pharmacological inhibition of iNOS on tubular cell death during renal inflammation *in vivo*. We used the neutrophil- and lymphocyte-independent model of experimental hydronephrosis that is characterized by prominent macrophage infiltration and significant tubular cell apoptosis.[22,26] Importantly, the administration of the specific iNOS inhibitor L-NIL did not result in any confounding effects on the level of interstitial macrophage infiltration. However, L-NIL treatment did significantly reduce the level of tubular cell apoptosis compared to the control group but had no effect on tubular cell proliferation, in accord with our *in vitro* experiments using primary murine tubular epithelial cells.

A

Control   L-NIL Treatment

B

Figure 8. Pharmacological blockade of iNOS increases tubulointerstitial deposition of collagen III after ureteric obstruction. Mice were administered L-NIL, an irreversible pharmacological inhibitor of the enzyme iNOS, or the isomeric control D-NIL, and the obstructed kidneys were removed at day 7. **A:** Photomicrographs of collagen III immunostaining at day 7 in control mice and mice treated with L-NIL. **B:** The administration of L-NIL increases the tubulointerstitial deposition of collagen III assessed by computer image analysis. *$P < 0.05$ versus control (n = seven to eight mice per group).

It should also be noted that additional mechanisms have been suggested for macrophage-mediated tubular cell apoptosis *in vivo*. A study of a murine model of Alport syndrome suggested that macrophage production of matrix metalloproteinases may result in degradation of tubular basement membrane and resultant tubular cell apoptosis by anoikis.[42,43] In addition, Misseri and colleagues[44] suggested a role for TNF-α in the induction of tubular cell death in experimental hydronephrosis in the rat. In this study, the obstructed kidneys exhibited up-regulation of both TNF-α and FasL expression with systemic administration of a pegylated form of soluble TNF receptor type 1, significantly inhibiting tubular cell apoptosis. Macrophage infiltration, however, was not determined, and it is therefore unclear whether blocking the action of TNF-α may have modulated macrophage infiltration, a key factor in determining the level of tubular cell apoptosis during tubulointerstitial infiltration.[14] However, because L-NIL administration resulted in a partial inhibition of tubular cell apoptosis, additional proapoptotic mediators may be used by infiltrating inflammatory macrophages to induce tubular cell death during tubulointerstitial inflammation *in vivo*.

Our study does, however, reinforce the critically important role of infiltrating macrophages during tubulointerstitial inflammation. A body of evidence indicates that macrophages are implicated in the deleterious induction of tubular cell apoptosis *in vivo*. For example, the induction of renal inflammation in mice exhibiting defective tubulointerstitial macrophage recruitment is characterized by significantly reduced levels of tubular cell apoptosis.[11–13] Also, we recently used the transgenic CD11b-DTR conditional ablation mouse[45] to examine the role of macrophages in progressive renal inflammation during nephrotoxic nephritis. The ablation of macrophages between days 15 and 20 of nephrotoxic nephritis improves renal function and significantly reduced the level of tubular cell apoptosis[14] thereby reinforcing a direct role for macro-

phages in the induction of tubular cell death. Our data indicate that the pharmacological blockade of the production of proapoptotic NO by iNOS can inhibit tubular epithelial cell death without affecting the level of macrophage infiltration and stresses the importance of macrophage phenotype as well as macrophage numbers during renal inflammation. Our data resonate with the work of Anders and colleagues[46] who noted that blockade of the chemokine CCL5/RANTES in glomerulonephritis successfully inhibited macrophage infiltration but resulted in increased macrophage expression of iNOS and increased tissue injury as a result.

It is also of interest that L-NIL treatment significantly reduced the level of interstitial cell apoptosis. Previous work has established that the vast majority of TUNEL-positive apoptotic cells detected within the tubulointerstitium of the kidney have actually been phagocytosed and are located within viable nonapoptotic cells.[47] We were therefore unable to use double staining for α-smooth muscle actin and TUNEL to determine whether the apoptotic tubulointerstitial cells were dying myofibroblasts. Despite the reduction in interstitial cell apoptosis, we found no significant difference in the tubulointerstitial population of myofibroblasts assessed by morphometric analysis of α-smooth muscle actin immunostaining. This may be attributable to the fact that the period of pharmacological blockade of iNOS was relatively short. Despite the absence of a significant difference in tubulointerstitial α-smooth muscle actin expression, we found that L-NIL treatment significantly increased the tubulointerstitial deposition of collagen III that is characteristically found in renal injury and scarring. This suggests that iNOS-derived NO may also be involved in limiting tubulointerstitial renal scarring and it is therefore of interest that iNOS knockout mice develop increased levels of tubulointerstitial fibrosis after ureteric obstruction.[48] Our data indicating that pharmacological blockade of iNOS-derived NO ameliorates the level of tubular cell apoptosis is not in accordance with previous work because NO has been

implicated as a protective factor in the induction of tubular cell apoptosis by mechanical stretch *in vitro* and *in vivo* in ureteric obstruction.[49] The reasons for this are unclear, although strain differences may be important because this can significantly affect the amount of NO generated by macrophages.[50] Although beyond the scope of this study, it would be interesting to perform ureteric obstruction in irradiated iNOS wild-type mice reconstituted with bone marrow derived from iNOS knockout mice because this would localize the defective expression of iNOS to the infiltrating leukocyte population. It should be noted, however, that double immunolabeling revealed F4/80-positive macrophages that were strongly iNOS-positive within the tubulointerstitium of obstructed kidneys, indicating that infiltrating macrophages may express iNOS.

Lastly, it is apparent from this and other studies that a significant level of macrophage-independent tubular cell death occurs in this model and it is undoubtedly the case that additional proapoptotic stimuli including mechanical stretch, hypoxia, ischemia, and so forth, also play a major role in the apoptosis of tubular epithelial cells.[51–53] In conclusion, our work indicates that iNOS-derived NO is an important mediator of macrophage-induced tubular epithelial cell apoptosis *in vitro* and plays an important role in tubular epithelial cell apoptosis and tubulointerstitial fibrosis *in vivo*.

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